*Abstract.* We have investigated proteins which interact with the PEST-type protein tyrosine phosphatase, PTP hematopoietic stem cell fraction (HSCF), using the yeast two-hybrid system. This resulted in the identification of proline, serine, threonine phosphatase interacting protein (PSTPIP), a novel member of the actin-associated protein family that is homologous to *Schizosaccharomyces pombe* CDC15p, a phosphorylated protein involved with the assembly of the actin ring in the cytokinetic cleavage furrow. The binding of PTP HSCF to PSTPIP was induced by a novel interaction between the putative coiled-coil region of PSTPIP and the COOH-terminal, proline-rich region of the phosphatase. PSTPIP is tyrosine phosphorylated both endogenously and in v-Src transfected COS cells, and cotransfection of dominant-negative PTP HSCF results in hyperphosphorylation of PSTPIP. This dominant-negative effect is dependent upon the inclusion of the COOH-terminal, proline-rich PSTPIP-binding region of the phosphatase. Confocal microscopy analysis of endogenous PSTPIP revealed colocalization with the cortical actin cytoskeleton, lamellipodia, and actin-rich cytokinetic cleavage furrow. Overexpression of PSTPIP in 3T3 cells resulted in the formation of extended filopodia, consistent with a role for this protein in actin reorganization. Finally, overexpression of mammalian PSTPIP in exponentially growing *S. pombe* results in a dominant-negative inhibition of cytokinesis. PSTPIP is therefore a novel actin-associated protein, potentially involved with cytokinesis, whose tyrosine phosphorylation is regulated by PTP HSCF.

THE control of cellular processes by tyrosine phosphorylation is a well-known aspect of eukaryotic physiology (Fantl et al., 1993; Hunter, 1994). While much information has accumulated regarding the functions of many tyrosine kinases, far less is understood about the physiological roles of protein tyrosine phosphatases (PTPs).[1] Approximately 50 PTPs have now been described, but the functions of just a handful are only beginning to be comprehended (Tonks, 1993; Dixon, 1996). In general, it appears that many of the PTPs are involved with the modulation of positive or negative signals induced by various tyrosine kinases. This function is most completely understood in the case of Src Homology (SH) PTP1, where mutations in the murine gene result in a number of hematopoietic abnormalities that are best explained by hyperactivity of diverse tyrosine kinases (Shultz, et al., 1993; Klingmuller et al., 1995). In another example, various members of the μ/κ/λ receptor PTP family may regulate the tyrosine phosphorylation levels of the cadherin–catenin complex, suggesting that these PTPs are involved with the control of cell adhesion (Brady-Kalnay et al., 1995; Fuchs et al., 1996; Cheng et al., 1997). The level of tyrosine phosphorylation of cyclin-dependent kinase is regulated by the CDC25 PTP, and this cyclical dephosphorylation is involved with the control of the cell cycle (Gautier et al., 1991). Finally, dual specific phosphatases, enzymes that are capable of dephosphorylating serine and threonine as well as tyrosine, may be involved with the regulation of MAP kinase phosphorylation, and are therefore critical for the regulation of disparate signaling phenomenon (Muda et al., 1996). While these data provide a number of compelling examples of the impor-

Please address all correspondence to L.A. Lasky, Department of Molecular Oncology, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080. Tel.: (415) 225-1123. Fax: (415) 225-6127. e-mail: lal@gene.com

1. *Abbreviations used in this paper*: CTH, COOH-terminal homology; GST, glutathione-S-transferase; HA, hemagglutanin; HSCF, hematopoietic stem cell fraction; HSP, hematopoietic specific protein; PSTPIP, proline, serine, threonine phosphatase interacting protein; PTP, protein tyrosine phosphatases.

tance of PTPs, it is likely that these enzymes are involved with a far greater diversity of cellular processes, which remain to be defined.

The PEST family of PTPs are a group of enzymes about which little functional information is known. The four examples of these enzymes, PTP PEST (Yang et al., 1993), PTP PEP (Matthews et al., 1992), PTP HSCF (Cheng et al., 1996) (also known as PTP-K1 [Huang et al., 1996], PTP20 [Aoki et al., 1996], fetal liver phosphatase (FLP)1 [Dosil et al., 1996]), and PTP brain-derived phosphatase (BDP)1 (Kim et al., 1996), contain an $NH_2$-terminal phosphatase domain followed by a variably sized region that is rich in proline, serine, and threonine. Initially, these noncatalytic COOH-terminal regions were thought to contain "PEST" motifs, which have been proposed to shorten intracellular protein half lives (Rogers et al., 1986). However, recent data have demonstrated that PEST PTPs do not appear to have extraordinarily short intracellular lifetimes (Flores et al., 1994; Charest et al., 1995), suggesting that these COOH-terminal regions may have other functions. Interestingly, the very COOH-termini of the PEST PTPs contain a 24–amino acid proline-rich region that is highly conserved in all four members of this family. Initially, it was proposed that this region was involved with the nuclear targeting of the PEP PTP (Flores et al., 1994), but subsequent data have demonstrated that this PTP (Cloutier et al., 1996), as well as PTP PEST (Yang et al., 1993; Charest et al., 1995), are both localized to the cytoplasm. In the case of PTP HSCF, one group has demonstrated that the enzyme is predominantly cytoplasmically localized (Huang et al., 1996), while another group demonstrated primarily nuclear localization using a different technique (Dosil et al., 1996). With respect to cell type expression, the PTP PEST is ubiquitously expressed (Yang et al., 1993); the PTP PEP is expressed in lymphoid cells (Matthews et al., 1992); the PTP HSCF is expressed in hematopoietic stem/progenitor cells and fetal thymus (Cheng et al., 1996; Dosil et al., 1996), as well as a subset of adult tissues, particularly, bone marrow (Huang et al., 1996); and the PTP BDP 1 is expressed at low levels in brain as well as other adult tissues (Kim et al., 1996).

The possible functions of PEST PTPs can be gleaned from an examination of the proteins that interact with these enzymes, the effects of overexpression of the phosphatases on cellular differentiation, and the possible modes of regulation of the molecules. Transfection of dominant-negative forms of PTP PEST into COS cells results in an endogenous, hyperphosphorylated protein that has been identified as $p130^{CAS}$, a cytoplasmic docking/adaptor-type molecule that contains an SH3 domain, as well as several potential tyrosine-phosphorylated SH2 binding sites (Garton et al., 1996). The function of $p130^{CAS}$ is incompletely understood, but it appears to be associated with focal adhesions and phosphorylated by the $p125^{FAK}$ (Petch et al., 1995) and the RAFTK (Astier et al., 1997) tyrosine kinases, suggesting that it may play a role in integrin-mediated signal transduction. Because dominant-negative PTP PEST inhibits dephosphorylation of $p130^{CAS}$, it is likely that this phosphoprotein is a substrate for this PTP, and it has been demonstrated that the recognition of $p130^{CAS}$ by PTP PEST is through the catalytic domain (Garton et al., 1996). It also has been shown recently that the PTB domain of the cytoplasmic adaptor protein SHC interacts with a nonphosphorylated, PTB-related binding site in the COOH-terminal region PTP PEST (Charest et al., 1996). Recent data have demonstrated that Csk, a cytoplasmic tyrosine kinase which inactivates Src family kinases, associates with the PEP PTP via an interaction between the Csk SH3 domain and one of the four proline-rich potential SH3 binding sites in the COOH-terminal region of the enzyme (Cloutier et al., 1996). Together, these results suggest that PTP PEST and PTP PEP both interact with critical cytoplasmic signaling proteins involved with the transmission of information from various cell surface receptors. Overexpression of PTP HSCF in PC12 cells resulted in a more rapid and robust neurite formation in response to NGF, suggesting that this PTP may be involved with cytoskeletal reorganization (Aoki et al., 1996). In support of this supposition, overexpression of a dominant-negative form of this enzyme in K562 hematopoietic progenitor cells resulted in an inhibition of cell spreading and substrate adhesion in response to phorbol ester (Dosil et al., 1996). With respect to regulation of these PTPs, analysis of PTP PEST revealed that phosphorylation of an $NH_2$-terminal serine residue (conserved in all members of the PEST PTP family) by protein kinase A resulted in the inhibition of phosphatase specific activity (Garton et al., 1994). These data suggest that serine, and potentially tyrosine phosphorylation of PEST PTPs may influence their catalytic activity.

We have used the yeast two-hybrid system to identify potential substrates for PTP HSCF. This has resulted in the isolation of a novel member of the actin associated protein family, termed proline, serine, threonine phosphatase interacting protein (PSTPIP), which is homologous to *Schizosaccharomyces pombe* CDC15, a phosphorylated, actin-associated protein involved with formation of the cleavage furrow during cytokinesis (Fankhauser et al., 1995). PSTPIP binds to PTP HSCF via a novel, high affinity interaction between the coiled-coil region of PSTPIP and the proline-rich COOH-terminal domain of the phosphatase. Endogenous PSTPIP is tyrosine phosphorylated in Baf3 cells and the v-Src tyrosine kinase induces the tyrosine phosphorylation of both PSTPIP as well as PTP HSCF. This phosphorylation is dramatically enhanced by coexpression of dominant-negative forms of PTP HSCF, suggesting that both of these proteins are substrates for the enzyme. The dominant-negative increase of PSTPIP tyrosine phosphorylation is dependent upon the inclusion of the COOH-terminal 24–amino acid PSTPIP binding site of the phosphatase. In Swiss 3T3 cells, endogenous PSTPIP is associated with both the cortical actin cytoskeleton and lamellipodia during interphase and also colocalizes with the actin ring of the cleavage furrow during cytokinesis. Overexpression of PSTPIP in Swiss 3T3 cells results in the formation of extended filopodial structures in a subset of the transfected cells, consistent with the possibility that this protein provokes actin reorganization. Interestingly, overexpression of PSTPIP in exponentially growing *S. pombe* results in a dominant-negative inhibition of cytokinesis. These data suggest that PSTPIP is a tyrosine-phosphorylated, cytoskeletal-associated protein, possibly involved with the control of cytokinesis, which is a substrate for the tyrosine phosphatase activity of PTP HSCF.

## Materials and Methods

### Two-hybrid Screening

Yeast two-hybrid screening was performed essentially as described in Chien et al. (1991) and Bartel et al. (1995). A $C_{221}$-S active site mutant of PTP HSCF (Cheng et al., 1996) was cloned in frame with the Gal binding domain in the plasmid pPC97. A library of $6 \times 10^6$ individual cDNA clones was produced from Baf3 lymphoid progenitor cells in the Gal activation domain plasmid, pPC86, using standard procedures. Yeast were transformed with both plasmids and were incubated on histidine minus plates for 3 d at 30°C. Colonies that grew under these conditions were restreaked onto histidine minus plates and were tested for β-galactosidase activity (Bartel et al., 1995). Colonies that manifested various levels of β-galactosidase activity were isolated, and the cDNA inserts in the pPC86 vector were isolated by PCR and sequenced using standard procedures. Clones encoding PSTPIP were tested for dependence on the PTP interaction by transfection into cells with and without the original PTP HSCF–containing pPC97 plasmid and subsequent analysis for growth on histidine minus plates and β-galactosidase activity.

### Mapping of Interaction Domains

To obtain a cDNA encoding full-length PSTPIP tagged with the FLAG epitope (DYKDDDDK) at the COOH terminus, PCR was performed using the 5′ PSTPIP–specific primer 48.BAMHI.F (CGCGGATCCACCATGATGGCCCAGCTGCAGTTC) and the 3′ PSTPIP–specific primer 48.SAL.FLAG.R (GTACGCGTCGACTCACTTGTCATCGTCGTCCTTGTAGTCGAGCTT). The resulting pcr fragment was digested with BamHI and SalI, and subcloned into the BamHI and SalI sites of pRK.tkneo, an expression plasmid containing the cytomegalovirus promoter, thus creating plasmid pRK.PIP.FLAG.C. The PTP HSCF deletion mutants were derived from a construct containing the influenza hemagglutinin epitope at its $NH_2$ terminus and were made as follows: PCR was performed on PRK.HSCF using primers prkr (TGCCTTTCTCTCCACAGG) and 38.SPE.mid.R (CTCCTTGAGGTTCTACTAGTGGGGGCTGGTGTCCTG). The resulting pcr fragment encoding the phosphatase domain (amino acids 1–302) was digested with ClaI and SpeI and subcloned into pRK.tk.neo digested with ClaI and XbaI resulting in plasmid pRK.HSCF.PTP domain. Similarly, a PTP HSCF cDNA missing the COOH-terminal homology (CTH) domain was produced by PCR using primers prkr and 39.SPE. endR (GCGGCCGCACTAGTATCCAGTCTGTGCTCCATCTGTTAC), and the resulting fragment encoding amino acids 1–430 of PTP HSCF was digested with ClaI and SpeI and subcloned into the ClaI and XbaI sites of pRKtkneo resulting in pRK HSCFΔ24. Glutathione-S-transferase (GST) fusion proteins were prepared essentially according to the manufacturer (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) in DH5-α bacterial cells. A SalI to NotI fragment containing the full-length cDNA for PSTPIP (amino acids 2–415) was subcloned into pGEX-4T-2 (Pharmacia LKB Biotechnology Inc.) cleaved at the SalI and NotI sites. To obtain a DNA fragment encoding the coiled-coil domain of PSTPIP, PCR was performed using primers PC86F (GCGTTTGGAATCACTAC) and pip48.1706R (TTATAGTTTAGCGGCCGCTCACCGGTAGTCCTGGGCTGATG). The PCR fragment was digested with SalI and NotI and subsequently cloned into the SalI and NotI sites of pGEX-4T-2. To obtain a cDNA fragment encoding the SH3 domain of PSTPIP, PCR was performed using primers pip48.1673.F (GTACGCGTCGACCGCACTCTACGACTACACTGCACAG) and PC86R (CTCTGGCGAAGAAGTCC), and the resulting product was digested with SalI and NotI and subcloned into the SalI and NotI sites of pGEX-4T-2. To obtain a cDNA fragment encoding the PST-rich region and CTH domain of PTP HSHCF (amino acids 304–453), PCR was performed using primers PST38-RI (GATCGAATTCCCAGAACCTCAAGGAGAACTGC) and PST38-XHOI (GATCCTCGAGTTACACCCGTGTCCACTCTGCTGGAGGA). The resulting pcr product was digested with EcoRI and XhoI, and then subcloned into the EcoRI and SaI sites of pGEX-4T-2. Protein determinations were carried out according to the Couprus assay with a kit from Geno Technology (St Louis, MO). The binding was carried out according to the method of Wong and Johnson (1996). Briefly, 1 μg of plasmid with either the PSTPIP protein or HSCF PTP under the control of the Sp6 promoter was in vitro–transcribed/translated using the Promega TnT Rabbit Reticulocyte system (Promega Corp., Madison, WI). Samples were diluted in 50 mM Hepes, pH 7.2, 1% Triton X-100, 10% glycerol, 100 mM NaCl, 5 mM EDTA, and 2 μm/ml each of leupeptin, pepstatin, aprotinin, and PMSF. Samples were precleared with resin for 1 h and 1 μg GST fusion protein was added along with 30 μl of GSH–Sepharose that was previously blocked in 3% BSA for 1 h. This was reacted for 1 h at 4°C and then the resin washed six times in Hepes/Triton X-100 binding buffer before SDS gel electrophoresis. The peptides were synthesized on an automated Milligen 9050 Peptide Synthesizer (Applied Biosystems, Inc., Foster City, CA) using standard solid phase chemistry with FMOC-protected amino acids on a p-alkoxybenzyl alcohol resin. Dried peptides were resuspended in the Hepes/Triton X-100 binding buffer at a concentration of 10 mg/ml. Peptide inhibition was performed by adding the peptide first to the in vitro translation product, and then to the GST fusion, followed by the GSH–Sepharose. The binding/washing steps were done as previously described. The peptides synthesized and the PTPs they were derived from were: PXXP-HSCF: $_{432}$GFNLRIGRPKGPRDPPAEWT$_{451}$ (PTP HSCF), PXXP-PEP:$_{782}$GFGNRFSKPKGPRNPPSAW$_{800}$ (PTP PEP), PXXP-PEST:$_{761}$GFGNRCGKPKGPRDPPSEWT$_{780}$ (PTP PEST), PXXP-CONTROL:$_{334}$GGVLRSISVPAPPTLPMADT$_{353}$ (PTP HSCF).

### Analysis of Tyrosine Phosphorylation

Baf3 or PSTPIP-transfected COS cells were lysed in 1% Triton X-100, 50 mM Hepes, pH 7.2, 10% glycerol, and 5 mM EDTA containing 1 μm/ml aprotinin, PMSF, leupeptin, and pepstatin with 1 mM sodium vanadate, and 10 mM iodoacetic acid. A portion of cells were pretreated with 0.1 mM pervanadate for 4 h before lysis. Immunoprecipitations were performed in the vanadate-containing lysis buffer using 1 μg/ml anti-PSTPIP polyclonal antibody and 400 μg of lysate protein at 4°C overnight. Western blots were performed using 1 μg/ml affinity-purified anti-PSTPIP or 2 μg/ml of commercial 4G10 anti-phosphotyrosine monoclonal (Upstate Biotechnology Inc., Lake Placid, NY). Signal was detected by ECL (Amersham Corp., Arlington Heights, IL) reagents (Pierce Chemical Co., Rockford, IL). The $C_{221}$-S mutant was as previously described (Cheng et al., 1996). The PTP HSCF $D_{197}$-A mutant was generated using PCR. Mutagenesis primer D197A.F (GTATATGTCCTGGCCAGCCCATGGGGTTCCCAGCAG), corresponding to nucleotide 591, and primer D197A.R (GCAGGTCGACTCTAGATTACACCCGTGTCCACTCTG), which corresponds to the stop codon, were used in PCR to generate a fragment that could be cut with MscI and XbaI. pRK.HA.38 WT, a plasmid that encoded the wild-type enzyme under the control of the cytomegalovirus promoter (Cheng et al., 1996), was digested with ClaI and MscI and the resulting 600-bp fragment was ligated with the MscI-XbaI pcr fragment into the ClaI and XbaI sites of pRK.tkneo. A plasmid encoding the v-Src oncogene under the control of the Sv40 early promoter was the kind gift of A. Levinson (Genentech, Inc., South San Francisco, CA). National Institutes of Health 3T3 cells and COS-7 cells were cultured in high glucose DME supplemented with 10% FBS, 2 mM L-Glutamine, 10 mM Hepes, pH 7.2, and pen-strep. COS-7 cells were transfected by electroporation. Briefly, $1.5 \times 10^{-6}$ COS-7 cells were mixed with 24 μg total DNA in PBS and electroporated at 960 microfarod, 0.22 V (Gene Pulsar; Bio Rad, Hercules, CA). After electroporation cells were seeded in 10-cm dishes and incubated for 3 d. 10-cm dishes of transfected COS cells were washed twice with ice-cold PBS, and then lysed in 1 ml of M-RIPA (50 mM Tris 7.4, 1% NP-40, 0.25% deoxycholate, 150 mM NaCl, 1 mM sodium ortho-vanadate, 1 mM NaF plus Complete™ Protease Inhibitors [Boehringer Mannheim Biochemicals, Indianapolis, IN]). Lysates were incubated for 15 min with 100 μl UltraLink Immobilized Protein A/G (Pierce Chemical Co.) at 4°C, followed by centrifugation for 5 min. Supernatants were collected and stored at −70°C, or directly immunoprecipitated. 5 μg of M2 (Eastman Kodak, Inc., Rochester, NY) or 12CA5 (Boehringer Mannheim, Inc.) was added to 500 μl of lysate and incubated overnight at 4°C. Ultralink Protein A/G was added and incubation continued for 2 h at 4°C. The immune complexes were washed three times with M-RIPA. The proteins were subjected to SDS-PAGE and transferred to nitrocellulose in 2× transfer Buffer, 20% methanol (Novex, San Diego, CA). Immunoblots were blocked at room temperature for 1 h in 3% milk/PBS. To detect FLAG-tagged PIP, blots were incubated overnight with 10 μg/ml Bio-M2 (Biotinylated anti-FLAG monoclonal Ab; Eastman Kodak), followed by incubation in 10 μg/ml streptavidin-HRP (Upstate Biotechnology Inc.). To detect hemagglutanin (HA)-tagged PTPhscf, blots were incubated in anti-HA-peroxidase (Boehringer Mannheim, Inc.) as per manufacturer's instructions. To detect phosphotyrosine, blots were incubated in HRP-conjugated 4G10 (anti-phosphotyrosine monoclonal; Upstate Biotechnology, Inc.) as per manufacturer's instructions.

oduced against a PSTPIP–GST fu-
-GST fusion protein was purified on
uscularly at two sites with 200 μg fu-
t multiple sites with a total of 300 μg
Complete Freunds Adjuvant (Sigma
abbits were boosted every 3 wk with
plete Freunds. 15 ml of rabbbit sera was
cted by centrifugation and washed with 10
-GSH-Sepharose for 3 h at 4°C with gen-
noglobulin was eluted from the affinity ma-
(X) mM NaCl, neutralized with NaOH, and
PBS. NIH 3T3 cells were seeded at 100,000
allowed to adhere overnight. The cells were
amine (2 μg pRK.PIP.FLAG.C/12 μl lipo-
MEM) for 5 h. The DNA/Lipofectamine solu-
h serum-containing medium added. 48 h after
the cells were fixed in 4% formaldehyde in
es. 25 mM Hepes, 10 mM EGTA, and 2 mM
hen permeabilized in 0.2% Triton X-100, 300 mM
r 10 min. The cells were washed twice in PHEM
with 10% FBS/PHEM 6.9 for 1 h to block nonspe-
tibody. Cells were incubated for 1 h in 2% BSA/
g 10 μg/ml M2 (anti-FLAG monoclonal antibody;
10 μg/ml 12CA5 (anti-HA monoclonal antibody;
im Biochemicals) as an irrelevant antibody control.
twice with 2% BSA/PHEM 6.9, cells were incubated
1:2,000) dilution of Cy3-conjugated AfinniPure sheep
nd a 1:200 dilution of fluorescein-phalloidin (Molecular
ne, OR) in 2% BSA/PHEM 6.9. Cells were washed in
6.9 and mounted in Vectashield Mounting Medium with
cells were seeded at 200,000 cells per chamber slide and
re overnight. Cells were stained with 0.4 μg/ml rabbit anti-
ml rabbit IgG and detected with Cy3-conjugated goat anti-
onally, cells were costained with a 1:200 dilution of fluores-
n. The samples were examined in a confocal microscope
Molecular Dynamics, Inc., Sunnyvale, CA); and analyzed
ageSpace software (Molecular Dynamics, Inc).

### *ion of PSTPIP in S. pombe*

e were grown according to standard methods (Moreno et al.,
cells were fixed and stained as for F-actin as described in Marks
ams (1985). The PSTPIP gene was expressed from *nmt 1* promoter
d. REP3 (Basi et al., 1993). For induction, a culture in mid-expo-
l growth in minimal medium containing 2 mM thiamine was grown
and then reinoculated into fresh medium without thiamine to in-
expression from the *nmt1* promoter at 25°C. Cell number was deter-
ed with a CASY-1 cell counter (Coulter Immunology, Hialeah, FL).

## *Results*

### *Identification of a PTP HSCF Binding Protein*

To identify potential substrates for PTP HSCF (Cheng et al., 1996), we performed a yeast two-hybrid screen using a catalytically inactive form of the enzyme as bait and a library derived from murine Baf3 hematopoietic progenitor cells, a cell type that has been previously demonstrated to express high levels of this phosphatase (Cheng et al., 1996). The catalytically inactive form of the enzyme was used to decrease the potential toxicity of the overexpressed protein for the cells. This resulted in the isolation of ~70 yeast clones that grew in the absence of histidine and expressed variable levels of β-galactosidase. Sequence analysis of the clones revealed that ~40% encoded related sequences with slightly divergent 5′ in-frame fusions with the Gal 4 activating domain. The sequences of the remainder of the clones suggested that they were likely due to ar-

struct in the
hybrid clone was used
the original Baf3 two-hybrid libra
Fig. 1 illustrates that the protein that inte
HSCF is a novel 415-residue molecule (predicted mo
lar weight of ~47,590) with significant sequence homology
to the *S. pombe* cell cycle protein, CDC15p, a cytoskeletal
interacting protein involved with organization of the actin
ring at the cleavage furrow during cytokinesis (Fankhauser
et al., 1995). This homology (~26% sequence similarity)
stretches over the entire length of both molecules, with the
exception of an insertion of ~500 residues in the yeast
molecule, and the yeast protein is the highest scoring ho-
mologue in the protein sequence database. A number of
features are conserved in these two proteins. For example,
both have an SH3 domain at their COOH termini (Paw-
son, 1995; Feng et al., 1995), and the mammalian SH3 do-
main appears to be homologous to those found in a num-
ber of known cytoskeletal regulatory proteins including
myosin heavy chain, spectrin, fodrin, hematopoietic spe-
cific protein (HSP) and cortactin (Fig. 1). In addition, both
the mammalian and yeast proteins contain a potential
coiled-coil domain at their $NH_2$ termini, which is predicted
both on the basis of sequence homology as well as an anal-
ysis of the mammalian sequence using the "Coil" program
(data not shown). Within these coiled-coil domains is a re-
gion with an extraordinary content of acidic and basic resi-
dues (positions 99–180 of the mammalian protein). Be-
cause the mammalian protein was isolated on the basis of
an interaction with a tyrosine phosphatase, it is possible
that the protein is tyrosine phosphorylated (see below).
and examination of the mammalian and yeast sequences
revealed seven conserved tyrosine residues (positions 53,
144, 191, 287, 363, 367, and 369 of the mammalian protein)
in addition to a number of non-conserved tyrosine residues.
Finally, examination of the proteins for proline-rich re-
gions that might function as SH3 binding sites (PXXP) re-
vealed one such conserved site in these proteins (starting
at position 278 of the mammalian protein) (Pawson, 1995;
Feng et al., 1995). Cortactin (Wu et al., 1991) and HSP 1
(Kitamura et al., 1989) are two other mammalian proteins
that contain potential coiled-coil and SH3 domains that
also bear a more distant relationship to the PTP-interact-
ing protein, although both these proteins contain homolo-
gous 37–amino acid repeats, which are absent from the in-
teracting protein. Because the mammalian sequence was
isolated based upon its ability to interact with the PEST
phosphatase PTP HSCF, it has been termed PSTPIP.

Northern blot analysis of the expression of PSTPIP dur-
ing embryogenesis and in adult tissues is illustrated in Fig
2. During embryogenesis, the transcript is most highly ex-
pressed in the day 7 embryo. The transcript is expressed at
relatively high levels in adult lung and spleen and at lower
levels in testis, muscle, kidney, brain, and heart. However,
the interacting protein is at far lower levels than actin,
since the actin blots were exposed for 4 h versus the 1 wk
exposure for the PSTPIP blots. Previously, we and others
have demonstrated that PTP HSCF is also expressed in

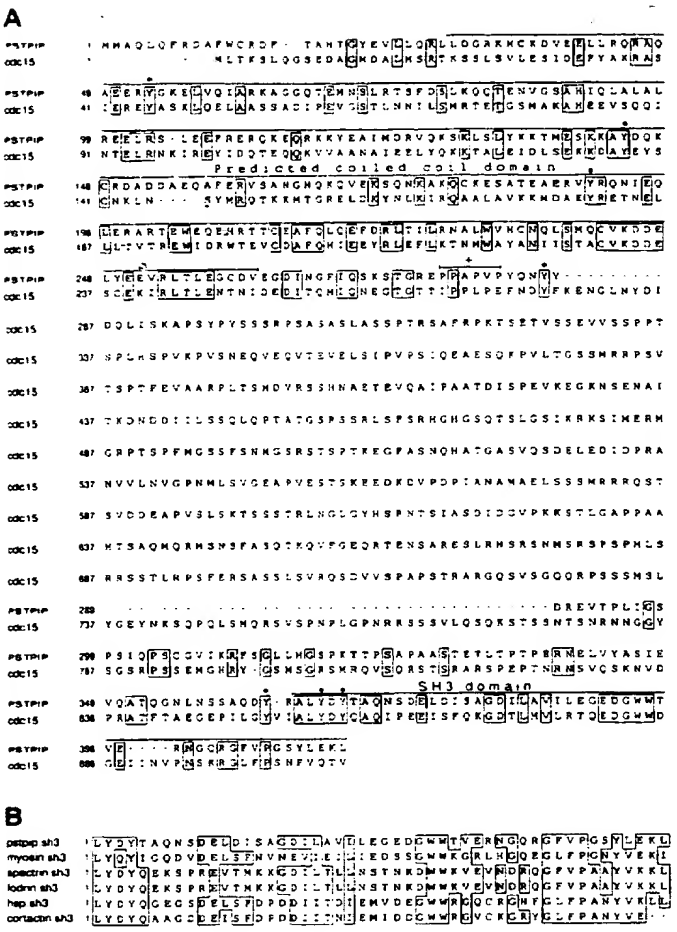


*Figure 1.* Protein sequence and putative domain structure of PSTPIP. (*A*) The comparison of the protein sequences of murine PSTPIP and *S. pombe* cdc15p. The asterisks illustrate the conserved tyrosine residues and the + shows the conserved potential SH3 binding site. The predicted coiled-coil and SH3 domains are overlined. (*B*) Sequence comparisons of the SH3 domains of PSTPIP and several different proteins known to interact with the cytoskeleton. (*C*) Domain structure of PSTPIP and cdc15p including the predicted coiled-coil regions containing regions rich in basic and acidic residues (+ −), the conserved tyrosines (*), the conserved potential SH3 binding site (†) and the conserved SH3 domains. Also shown is the large region in the *S. pombe* protein that contains predicted PEST degradation signals and which is missing from the mammalian homologue. These sequence data are available from EMBL/GenBank/DDBJ under accession number U87814.

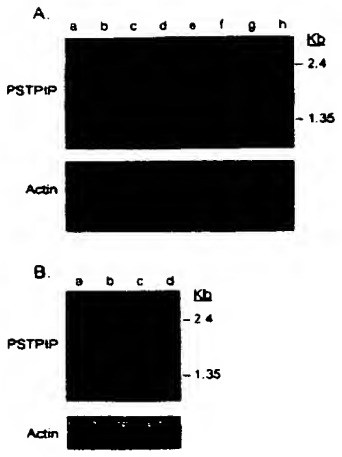


*Figure 2.* Northern blot analysis of the expression of PSTPIP transcript. (*A*) Expression of PSTPIP and actin in heart (*a*), brain (*b*), spleen (*c*), lung (*d*), liver (*e*), muscle (*f*), kidney (*g*), and testis (*h*). (*B*) Expression of PSTPIP and actin in 7 (*a*), 11 (*b*), 15 (*c*), and 17-d (*d*) murine embryos.

both adult lung and kidney (Cheng et al., 1996; Huang et al., 1996).

### *Characterization of the Interaction between PTP HSCF and PSTPIP*

To characterize the regions involved with the binding between PTP HSCF and PSTPIP, a rapid and direct in vitro binding assay was performed. In this assay, various GST fusions of either the phosphatase or the interacting protein were used to precipitate in vitro translation products of the cognate binding proteins. Fig. 3 illustrates that precipitation of in vitro–translated PTP HSCF by GST fusion proteins containing various SH3 domains as well as full-length PSTPIP demonstrated a high degree of specificity in the interaction between the GST PSTPIP and the phosphatase. The figure also illustrates that at this concentration of GST fusion protein (~1 μg/ml or ~1.5 μM), the PSTPIP fusion protein appeared to be more efficient at precipitating the phosphatase than a polyclonal antibody directed against the enzyme or a monoclonal directed against a hemagglutinin tag at the PTP $NH_2$ terminus (data not shown). This result is consistent with a relatively high affinity interaction between the GST PSTPIP and the in vitro–translated PTP HSCF.

The region of PTP HSCF that interacts with PSTPIP was identified by producing deletion mutants of the enzyme missing either the 24–amino acid CTH domain, which is highly conserved in all of the PEST PTPs (Matthews et al., 1992; Yang et al., 1993; Aoki et al., 1996; Cheng et al., 1996; Dosil et al., 1996; Huang et al., 1996; Kim et al., 1996) or both this domain as well as the longer proline, serine, threonine (PST)-rich region COOH terminal to the catalytic domain. Fig. 4 reveals that deletion of the COOH-terminal 24–amino acid homology domain of PTP HSCF completely abolished the interaction between these two proteins. Because this region is conserved in all PEST PTPs, it is possible that both PTP PEST (Yang et


*Figure 3.* Interaction between PTP HSCF and GST-PSTPIP. Precipitations of in vitro–transcribed and -translated PTP HSCF phosphatase with: GST-p85 SH3 (*a*); GST alone (*b*); GST-Src SH3 (*c*); GST-Grb2 N-SH3 (*d*); GST-PSTPIP (*e*); GST-Abl SH3 (*f*); GST-PLC SH3 (*g*); anti-PTP HSCF (*h*) polyclonal antibody; and GST-Spectrin SH3 (*i*).

al., 1993) as well as PTP PEP (Matthews et al., 1992) also interact with PSTPIP. To examine this possibility, and also to examine if the COOH terminal region is sufficient for this interaction, 20-residue long peptides derived from the homologous COOH terminal domains of three PEST PTPs were used to compete with the interaction between PTP HSCF and PSTPIP. In this form of the assay, a GST fusion derived from the PST-rich and COOH CTH regions of the phosphatase was used to precipitate in vitro–translated PSTPIP in the presence of varying amounts of peptides (Fig. 4 *C*). Fig. 4 illustrates that all three peptides effectively block the interaction at concentrations as low as ~800 nM, while a control peptide derived from a different proline-rich region of PTP HSCF is unable to block the interaction. These data suggest that this small proline-rich region of the PEST PTPs is sufficient for mediating the high affinity interaction between the phosphatase and PSTPIP, and furthermore indicate the possibility that all of these PTPs may interact with PSTPIP via their CTH domains.

To examine the region of PSTPIP that interacts with the COOH-terminal homology region, GST fusions containing either the SH3 domain or the coiled-coil domain of the interacting protein were used to immunoprecipitate in vitro–translated PTP HSCF. The COOH terminal homology region that interacts with PSTPIP contains two overlapping consensus SH3 (PXXP) binding sites, consistent with the possibility that the phosphatase-PSTPIP interaction was an SH3-type binding event (Pawson, 1995; Feng et al., 1995). However, the affinity of the interaction as measured in the peptide experiment described above was significantly greater than many of those previously reported for SH3 domain–PXXP interactions (Feng et al., 1995), and as Fig. 5 illustrates, the interaction between these proteins was surprisingly mediated by the coiled-coil domain and not the SH3 region. This outcome is consistent with the results of the two-hybrid clones, all of which began at a site very close to the $NH_2$ terminus of the coiled-coil domain, suggesting that the PSTPIP site that interacts with the COOH terminal proline-rich domain requires the $NH_2$ terminus. Finally, Fig. 5 also illustrates that these two proteins interact in vivo in transfected COS cells, confirming the in vitro binding data. Thus, these data define a novel high affinity in vivo interaction between the COOH-terminal proline-rich domain of PTP HSCF, and the potential coiled-coil domain of PSTPIP.

### *PSTPIP Is a Substrate for PTP HSCF Phosphatase Activity*

The association between PTP HSCF and PSTPIP suggested that the interacting protein might be a substrate for

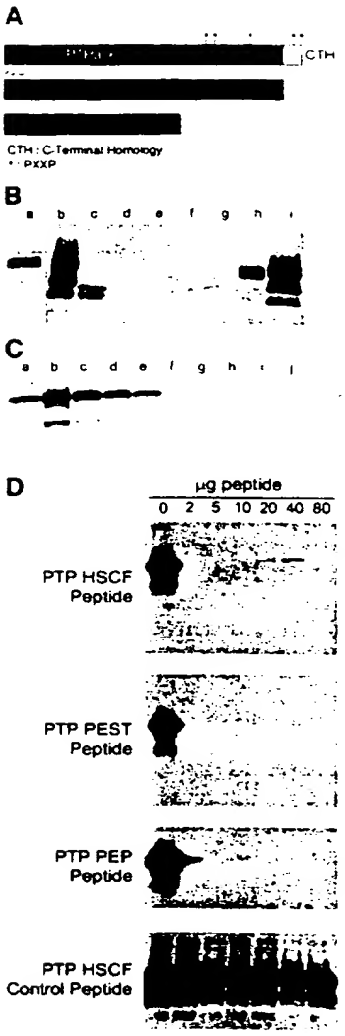


*Figure 4.* Mapping of the PSTPIP interaction site on PTP HSCF. (*A*) PTP HSCF constructs containing full-length, CTH and PST-rich domain deletion mutants used for in vitro transcription and translation were precipitated with a GST fusion protein containing the full-length PSTPIP sequence. (*B*) Precipitation of in vitro–transcribed and -translated forms of PTP HSCF with GST-PSTPIP or anti-PTP HSCF polyclonal antibody: full-length PTP HSCF with anti-PTP HSCF polyclonal antibody (*a*); full-length PTP HSCF with GST-PSTPIP (*b*); PST-rich + CTH-deleted PTP HSCF with anti-PTP HSCF polyclonal antibody (*c*); PST-rich + CTH deleted PTP HSCF with GST-PSTPIP (*d*); PST-rich + CTH-deleted PTP HSCF with GST-Spectrin SH3 domain (*e*); CTH-deleted PTP HSCF with GST-Spectrin SH3 domain (*f*); CTH-deleted PTP HSCF with GST-PSTPIP (*g*); CTH-deleted PTP HSCF with anti-PTP HSC polyclonal antibody (*h*); full-length PTP HSCF with anti-PTP HSCF polyclonal antibody (*i*). (*C*) Precipitation of in vitro–transcribed and -translated full-length PSTPIP with anti-PSTPIP polyclonal antibody (*a*) or 10 μg (*b*); 5 (*c*); 2 (*d*); or 1 μg (*e*) of GST-PST-rich + CTH PTP HSCF (a GST construct containing the PST-rich and CTH domains including amino acids 304–453 of the phosphatase), preimmune antibody (*f*), or 10 (*g*), 5 (*h*); 2 (*i*) or 1 μg (*j*) of GST alone. (*D*) Precipitation of in vitro–transcribed and -translated PSTPIP with GST-PST-rich + CTH PTP HSCF in the presence of decreasing amounts of proline-rich peptides derived from the COOH-terminal homology regions of PTPs HSCF, PEST, and PEP or a control proline-rich peptide from PTP HSCF (see Materials and Methods for peptide sequences).

the phosphatase. In addition, the conservation of a number of tyrosines between PSTPIP and the phosphorylated CDC15 protein was also consistent with the possibility that the interacting protein was tyrosine phosphorylated. As Fig. 6 *A* demonstrates, endogenous PSTPIP is indeed tyrosine phosphorylated in Baf3 cells in the presence of the tyrosine phosphatase inhibitor vanadate (Dixon, 1995), consistent with the supposition that the protein is dephosphorylated in vivo by a PTP, possibly PTP HSCF which is coexpressed in these cells (Cheng et al., 1996). In addition, tyrosine phosphorylation of transfected PSTPIP can also be demonstrated to occur in COS cells in the presence of vanadate (Fig. 6 *A*). A potential tyrosine kinase that might

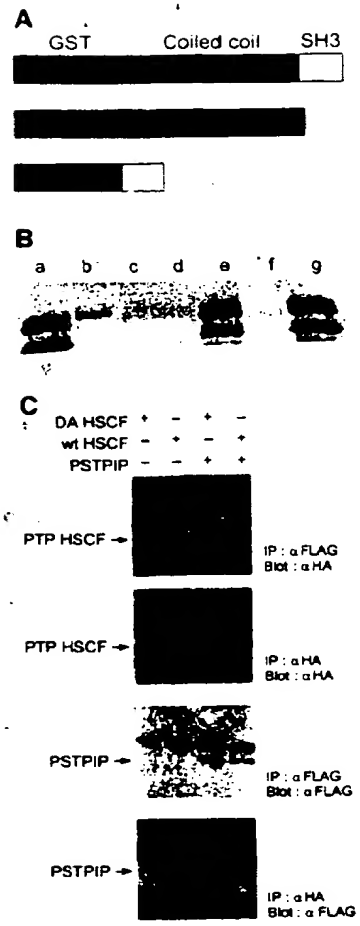


*Figure 5.* Mapping of PTP HSCF interaction site on PSTPIP. (*A*) GST fusions containing the full-length, coiled-coil, or SH3 domains of PSTPIP were used to precipitate in vitro–transcribed and -translated full-length PTP HSCF. (*B*) Precipitation of full-length PTP HSCF with: GST full-length PSTPIP (*a*); anti-hemagglutinin monoclonal antibody (*b*) (directed against a hemagglutinin epitope tag at the $NH_2$-terminus of the PTP HSCF); GST-Grb 2 SH3 domain (*c*); GST-Spectrin SH3 domain (*d*); GST full-length PSTPIP (*e*); GST-SH3 PSTPIP (*f*); and GST–coiled-coil PSTPIP (*g*). (*C*) Cos cells were transfected with the indicated plasmids containing hemagglutinin (*HA*)-tagged, wild-type (*wt HSCF*), or dominant-negative (*DA HSCF*) forms of PTP HSCF and FLAG-tagged PSTPIP. Precipitation of PSTPIP (anti-FLAG-tagged) brings down PTP HSCF (anti-HA tagged) and precipitation of PTP HSCF (anti-HA tagged) brings down PSTPIP (anti-FLAG tagged).

phosphorylate PSTPIP in vivo is one of the Src family kinases. Previous data suggested that the v-Src tyrosine kinase is associated with the cytoskeleton, modulates cytoskeletal elements that resulted in profound morphological

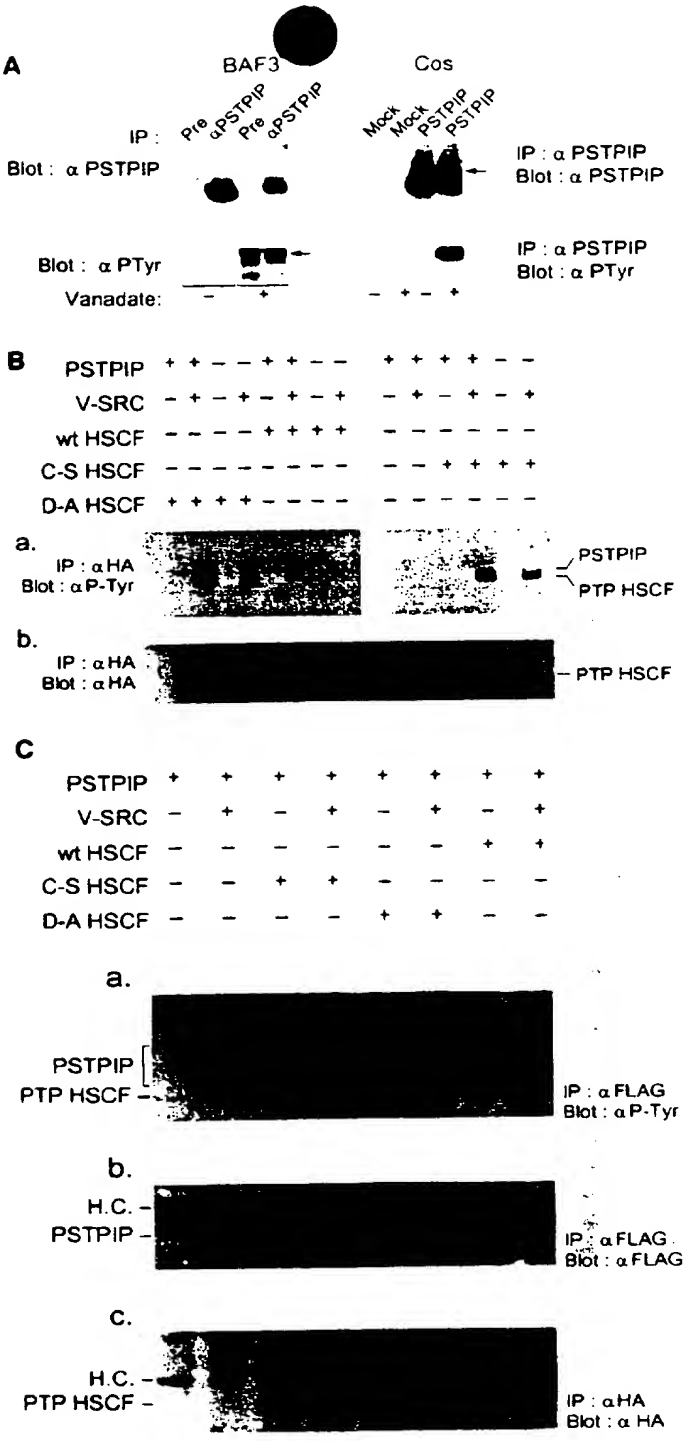


*Figure 6.* In vivo tyrosine phosphorylation of PSTPIP. (*A*) The left panel shows the immunoprecipitation of endogenous PSTPIP from Baf3 cells (*BAF3*) with anti-PSTPIP polyclonal antibody in control cells or cells pretreated with the PTP inhibitor pervanadate. Precipitates were blotted with either anti-PSTPIP or anti-phosphotyrosine antibodies. Note that the protein in the absence of pervanadate is lower molecular weight and not tyrosine phosphorylated. The right panel illustrates the results of pervanadate treatment in mock-transfected or PSTPIP transfected COS cells (*Cos*). The transfected PSTPIP is tyrosine phosphorylated in cells pretreated with pervanadate, although the stoichiometry of phosphorylation appears less than in Baf3 cells. (*B*) Immunoprecipitations and blots were done with the indicated antibodies on COS cells transfected with the combinations of plasmids shown at the top of the figure. (*a*) Immunoprecipitation of PTP HSCF with anti-HA antibody directed against an $NH_2$-terminal HA epitope and blotted with anti-phosphotyrosine. Note that both PSTPIP as well as PTP HSCF are highly tyrosine phosphorylated in the presence of the dominant-negative PTP mutants (*C-S* and *D-A*), but not in the presence of the wild type (*wt*) PTP. (*b*) Immunoprecipitation of PTP HSCF with anti-HA antibody and blotting with anti-HA antibody. (*C*) Immunoprecipitations and blots were done with the indicated antibodies on COS cells transfected with the combinations of plasmids shown at the top of the figure. (*a*) Immunoprecipitation of PSTPIP with anti-FLAG antibody directed against a COOH-terminal FLAG epitope on PSTPIP and blotting with anti-phosphotyrosine. Note that both the coprecipitated PTP HSCF as well as PSTPIP are hyperphosphorylated in the presence of dominant-negative PTPs. (*b*) Immunoprecipitation of PSTPIP with anti-FLAG antibody and blotting with anti-FLAG. The absence of visible protein in the lanes containing highly tyrosine phosphorylated PSTPIP may be due to phosphorylation of the tyrosine in the FLAG epitope. The proteins are clearly visible in the anti-phosphotyrosine blot, however. (*c*) Immunoprecipitation of PTP HSCF with anti-HA antibody directed against an $NH_2$-terminal hemagglutinin epitope and blotting with the same antibody.

changes (Cooper et al., 1993; Kaplan et al., 1994; Thomas et al., 1995), and mediates the tyrosine phosphorylation of cortactin (Wu et al., 1991; Okamura et al., 1995; Vuori et al., 1995; Dehio et al., 1995), an SH3-, coiled-coil–containing actin binding protein that bore a distant structural similarity to PSTPIP. In addition, HSP 1, another SH3-containing protein that is also structurally similar to PSTPIP, is tyrosine phosphorylated by various Src family kinases (Yamanashi et al., 1993; Nada et al., 1994; Takemoto et al., 1995; Takemoto et al., 1996). These results implied that v-Src, a constitutively active form of the enyzme, might mediate the tyrosine phosphorylation of PSTPIP, thus allowing for an analysis of the possible substrate interactions between the interacting protein and PTP HSCF. To test this possibility, PSTPIP was transfected into COS cells together with the v-Src tyrosine kinase and either wild-type or dominant-negative forms of PTP HSCF. Dominant-negative phosphatases were produced by mutating either the active site cysteine to a serine ($C_{229}$-S), which abolishes the ability of the enzyme to form a covalent transition state intermediate with the phosphate attached to the tyrosine, or by mutating a critical active site aspartate residue to alanine ($D_{197}$-A), which inhibits the catalytic removal of the phosphate (Dixon, 1995; Jia et al., 1995; Garton et al., 1996). In both cases, these mutants will tightly bind to the substrate but not dephosphorylate it, with the result that the substrate will be hyperphosphorylated. This procedure has been previously used to characterize substrates for a number of different PTPs, including PTP PEST (Garton et al., 1996) and PTP SHP-2 (Herbst et al., 1996) and it has revealed that these mutant enzymes show exquisite substrate specificity in vivo.

As can be seen from Fig. 6, *B* and *C*, both PTP HSCF as well as PSTPIP are tyrosine phosphorylated in response to v-Src cotransfection. Transfection of wild-type, C-S, and D-A mutants of PTP HSCF together with v-Src (Fig. 6 *B*) demonstrates that the mutant forms of the enzyme were hyperphosphorylated, while the wild-type PTP was not; this is consistent with the enzyme being a substrate for v-Src tyrosine phosphorylation. In addition, because only the dominant-negative forms of the enzyme mediated PTP HSCF hyperphosphorylation, these results also suggest that PTP HSCF is a substrate for its own catalytic activity. Fig. 6 *B* also illustrates that PSTPIP coprecipitates with the dominant-negative forms of the enzyme and is hyperphosphorylated, consistent with the conclusion that PSTPIP is a substrate for the catalytic activity of PTP HSCF. Fig. 6 *C* illustrates that transfection of the wild-type PTP HSCF into PSTPIP and v-Src–expressing cells resulted in a decreased level of tyrosine phosphate on PSTPIP, consistent with the in vivo removal of the phosphate from PSTPIP tyrosines by the phosphatase, a result that would be expected if the interacting protein were a substrate for the enzyme. Even more compellingly, Fig. 6 *C* also illustrates that cotransfection of either dominant-negative form of PTP HSCF into PSTPIP and v-Src–transfected cells resulted in a dramatic increase in the levels of tyrosine phosphate on the interacting protein as well as on the coprecipitating PTP HSCF. Analysis of total tyrosine-phosphorylated proteins in transfected cells demonstrated that the dominant-negative forms of PTP HSCF showed complete substrate specificity for both PTP HSCF itself

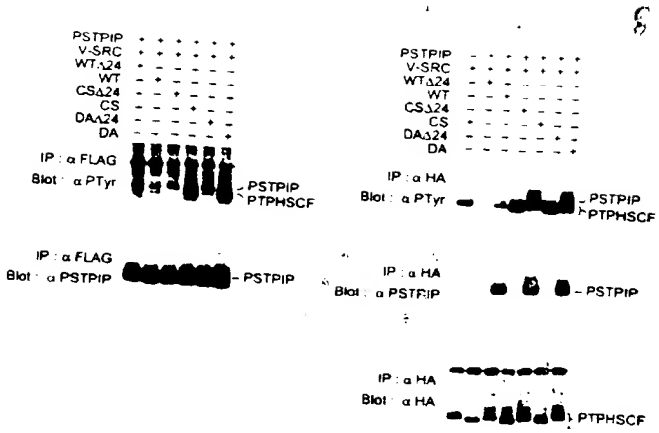


*Figure 7.* Analysis of PTP HSCF mutants with deletions of the 24–amino acid COOH-terminal homology domain. Immunoprecipitations and blots were done with the indicated antibodies on COS cells transfected with plasmids shown at the top of the figure. The left panels illustrate the immunoprecipitation of FLAG-tagged PSTPIP, while the right panels show the immunoprecipitation of HA-tagged PTP HSCF. The mutants with deletions of the COOH-terminal 24–amino acid homology domain are shown as *WT*, wild type; *CS*, $Cys_{229}$-Ser; and *DA*, $Asp_{197}$-Ala Δ24. Note that hyperphosphorylation of PSTPIP by the dominant-negative PTPs only occurs when the COOH-terminal 24–amino acids are included in the proteins. In addition, note that PSTPIP only coprecipitates with forms of PTP HSCF that include the COOH-terminal 24–amino acid homology domain.

and PSTPIP (data not shown). These data thus confirm the conclusion that PSTPIP interacts with PTP HSCF in vivo, and they suggest that this interaction allows the phosphatase to dephosphorylate tyrosine residues modified by the v-Src kinase. Moreover, they imply that PTP HSCF is a v-Src substrate for tyrosine phosphorylation, and that these phosphotyrosine residues are substrates for the catalytic activity of the phosphatase. In addition, because tyrosine-phosphorylated PSTPIP was only observed in cells that were transfected with v-Src, these results also suggest that the dramatic overexpression of the protein in these cells may have overwhelmed the endogenous tyrosine phosphorylation mechanism.

The in vitro mapping analysis described previously suggested that PSTPIP interacted with PTP HSCF via the COOH-terminal 24–amino acid homology domain found in all PEST PTPs. Previous work in the PEST PTP system demonstrated that $p130^{cas}$ was a substrate for this enzyme, and the recognition of this substrate appeared to be predominantly dependent upon the catalytic domain (Garton et al., 1996). To test for the dependence of the PTP HSCF CTH domain on PSTPIP substrate recognition, wild-type and dominant-negative forms of the enzyme were produced that lacked the COOH terminal 24 amino acids. These deletion mutants were then tested for their ability to hyperphosphorylate PSTPIP in the presence of v-Src. Fig. 7 illustrates that the deletion of the COOH CTH domain of the two catalytically inactive, dominant-negative PTPs resulted in a complete lack of hyperphosphorylation of PSTPIP in the presence of v-Src. In addition, these experiments confirm the in vitro mapping studies by demonstrating that PSTPIP cannot interact with forms of PTP

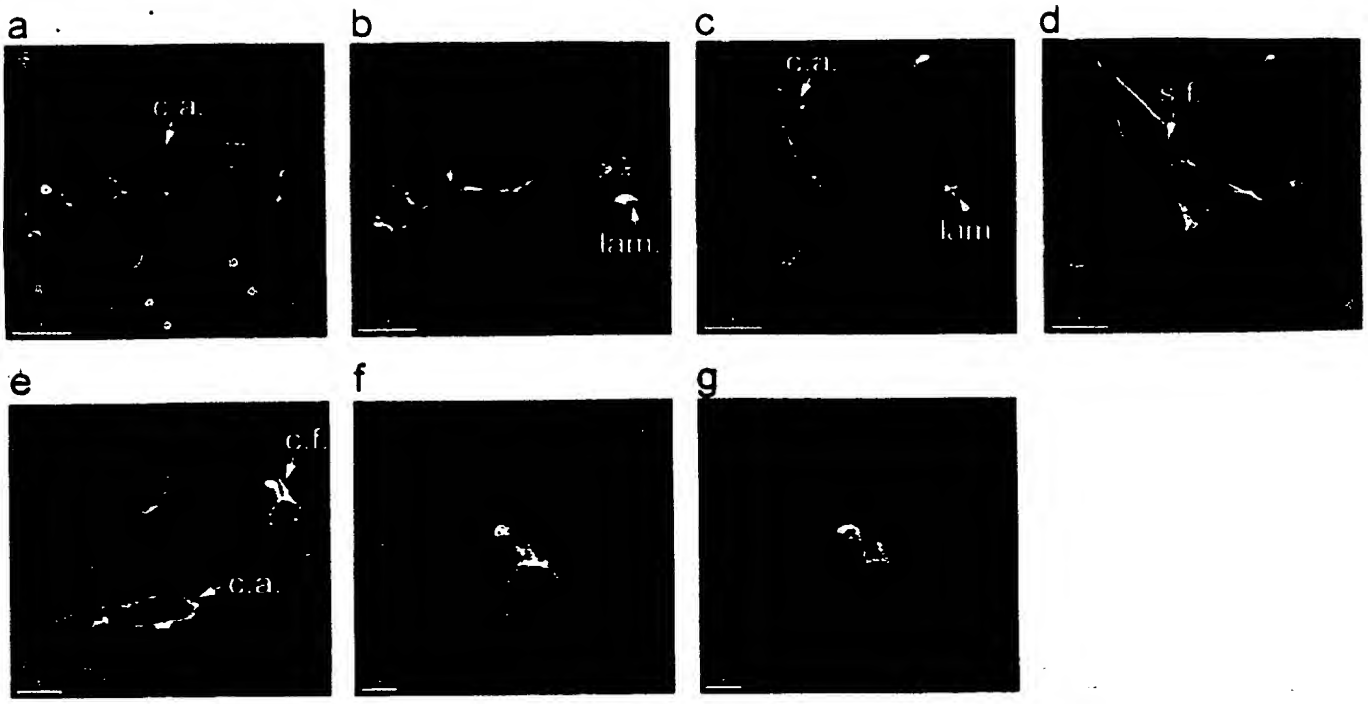


*Figure 8.* Localization of endogenous PSTPIP in 3T3 cells. Confocal images of two different groups of 3T3 cells viewed at different focal planes stained with affinity-purified anti-PSTPIP polyclonal antibody (Cy3 labeled) and phalloidin-FITC (*a–d*). Sites of colocalization appear yellow and are the cortical actin (*c.a.*), the lamellipodia (*lam.*), and the stress fibers (*s.f.*). *e* and *f* illustrate a low magnification and high magnification views of interphase cells and cells undergoing cytokinesis stained with the same reagents. The interphase cells show colocalization predominantly in the cortical actin (*c.a.*) region at this focal plane, while the cells undergoing cytokinesis show colocalization predominantly at the cleavage furrow (*c.f.*) at both focal planes shown. Bars: (*a* and *b*) 10 μm; (*c* and *d*) 20 μm; (*e*) 5 μm; (*f* and *g*) 2 μm.

HSCF that are missing the COOH CTH domain. These data thus indicate that the interaction between PSTPIP and the dominant-negative forms of PTP HSCF that mediates the hyperphosphorylation of the interacting protein is not due to the catalytic domain, as is the case for the hyperphosphorylation of $p130^{cas}$ by dominant-negative PEST PTPs, but is instead induced by the interaction between the coiled-coil domain and the COOH-terminal homology region.

### *Subcellular Localization of PSTPIP*

*S. pombe* CDC15p is associated with the cleavage furrow localized F-actin during cytokinesis (Fankhauser et al., 1995). To analyze the subcellular localization of endogenous PSTPIP, 3T3 cells were stained with an affinity-purified polyclonal antibody directed against a GST fusion of the protein and were imaged using confocal microscopy. Fig. 8 illustrates that the interacting protein is colocalized to several F-actin–containing sites in the cell. A majority of the protein appears to be associated with the cortical actin cytoskeleton. The protein also appears to colocalize with the actin stress fibers as well as in lamellipodial regions of the cell. In addition, transfection of PSTPIP into CHO cells revealed expression at sites of focal contact (data not shown). These results are in contrast with the PSTPIP-related protein cortactin, which shows localization on cortical actin and at the ends of the stress fibers but not the fibers themselves (Wu et al., 1991). These data clearly suggest that PSTPIP is associated with cytoskeletal actin during interphase.

Importantly, examination of cells undergoing cytokinesis reveals that endogenous PSTPIP is predominantly associated with the cleavage furrow (Fankhauser et al., 1995; Fishkind and Wang, 1995). As Fig. 8 shows, both PSTPIP and the actin ring colocalize to this region of the dividing cells. This figure also illustrates that the PSTPIP in the cleavage furrow is predominantly associated with the membrane-bound F-actin, which acts to constrict the cleavage furrow (Fishkind et al., 1995), and examination of sections taken perpendicular to the cleavage furrow support this, showing a donutlike structure containing both PSTPIP and actin attached to the constricting plasma membrane of the cleavage furrow (data not shown). It also appears from this figure that much of the cortically associated actin and PSTPIP migrate to the cleavage furrow during cytokinesis, a result that is similar to that observed for yeast CDC15p and actin (Fankhauser et al., 1995). These subcellular localization data are thus consistent with the conclusion that PSTPIP is an actin binding protein that is potentially involved with the regulation of the cleavage furrow.

### *Filopodial Induction by Overexpressed PSTPIP*

One role that PSTPIP might play in the cleavage furrow is the reorganization of polymerized actin (Cao et al., 1990*a*;

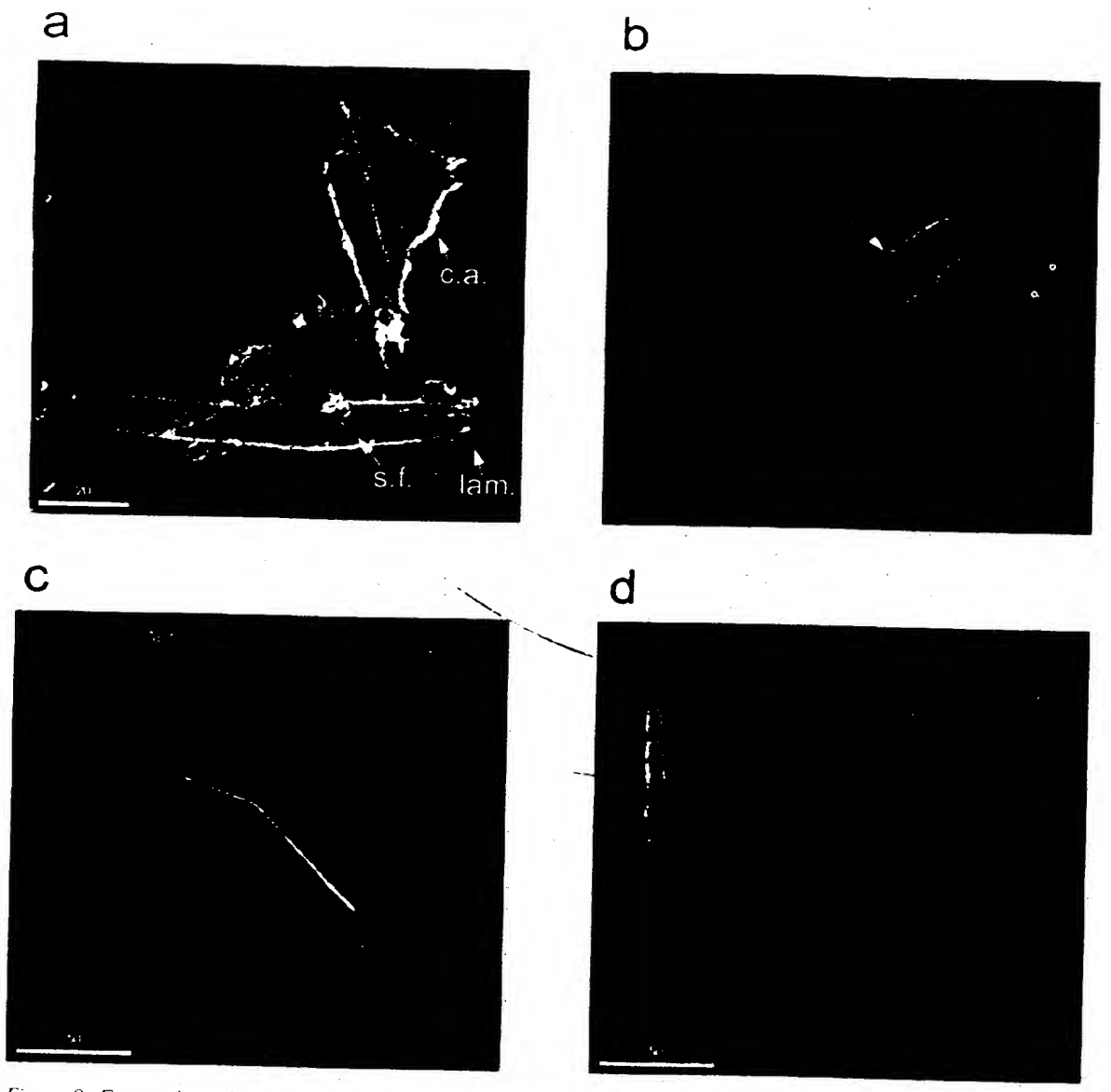


*Figure 9.* Expression of PSTPIP in transfected 3T3 cells. (*a*) A group of 3T3 cells transfected with an expression plasmid containing a COOH-terminal FLAG version of PSTPIP under the control of the strong cytomegalovirus promoter. Cells were stained with anti-FLAG antibody (Cy3 labeled) and phalloidin-FITC. PSTPIP colocalizes with actin at the cortical region (*c.a.*), the stress fibers (*s.f.*), and the lamellipodia (*lam.*). (*b*) A 3T3 cell contransfected with PSTPIP-FLAG and HA-PTP HSCF, and stained with anti-FLAG (Cy3-labeled) and anti-HA (FITC-labeled). Note the colocalization of staining at the cell's cortex (*arrowhead*). (*c* and *d*). Two cells with abnormal morphology expressing PSTPIP. Note that these filopodial structures are greater than 100 μm in length. Bars: (*a* and *b*) 20 μm; (*c* and *d*) 50 μm.

Fishkind and Wang, 1993, 1995). To examine the possible function of PSTPIP in actin assembly, 3T3 cells were transfected with an epitope-tagged version of the protein under the control of the powerful cytomegalovirus promoter, and the transfected cells were subsequently examined for expression of transfected PSTPIP as well as F-actin. As can be seen in Fig. 9, 3T3 cells with normal morphology that expressed transfected PSTPIP showed colocalization of the protein at the cortical surface with F-actin as well as in lamellipodial structures and the F-actin stress fibers; this is in agreement with data obtained examining endogenous PSTPIP localization (Fig. 8). In addition, this figure shows that PSTPIP colocalizes with PTP HSCF in cotransfected 3T3 cells. Fig. 9 also illustrates that the overexpression of the protein often induced a remarkable morphological change in a high percentage of 3T3 cells. These cells contained extended, filopodial-like structures that were filled with polymerized actin. In many cases, the structures were up to ~150 μm in length, and they often showed a knob-like morphology. In addition, the majority of cells contained a single extended filapodial structure. It appears that this structure was probably produced in the absence of significant cell growth or plasma membrane synthesis, since the overall size of the cell body appeared to decrease

dramatically concomitant with the lei ting of the filapodial structure. This type of cell mo ogy is never observed with transfection of the green fluorescent protein (data not shown), and Fig. 9 illustrates that it is very different from the morphology of normally elongated, nontransfected cells. In summary, these results suggest that the unregulated expression of PSTPIP in vivo results in the induction of extended filopodial-like structures, consistent with the possibility that the overexpressed protein may induce an inappropriate polymerization of the cortical cytoskeleton.

### *Overexpression of PSTPIP in Exponentially Growing S. pombe*

To examine the function of PSTPIP in a readily controlled, genetically defined in vivo system, we overexpressed the protein in the fission yeast *S. pombe* using the thiamine inducible promoter system (Basi et al., 1993). Initial studies demonstrated that the mammalian protein was incapable of rescuing previously described CDC15 null mutants (Fankhauser et al., 1995). After induction of PSTPIP expression in wild-type cells, cell number increased more slowly than in nonexpressing cells, but division did not cease. The doubling time increased from 3 to 3.5 h. However, we noted a significant increase in the number of septated cells one generation after induction (the promoter takes three and one-half generations to induce). The number of septated cells increased from ~12% in the uninduced control, to ~36% (Fig. 10, *5 gen*). At later times, a second phenotype appeared: cells did not cleave, but resumed growth from the tips, becoming elongated (Fig. 10, *6 gen, right-hand cell*). These cells then formed a pair of septa after mitosis, producing a cell with three septa separating four nuclei (Fig. 10, *6 gen, left-hand cell*). Approximately one-third of [redacted]eptated cells displayed this phenotype. The block to [redacted]cleavage was not absolute, since the number of septated cells did not exceed 36%, cell number continued to increase, though at a reduced rate, and cells were seen to cleave (Fig. 10, *8 gen, right-hand cell*). Actin staining of cells overexpressing PSTPIP showed that in interphase, it is seen as dots at the tips, while in mitosis, it forms a ring, then associates with the growing septum, as expected (data not shown). FLAG staining for PSTPIP showed that the epitope localizes to the tips in interphase and to the cell equator at mitosis (Fig. 10). It thus has very similar localization to actin, in support of the data obtained for PSTPIP localization in mammalian cells. These data are thus consistent with the suggestion that mammalian PSTPIP acts as a dominant-negative inhibitor of cytokinesis in *S. pombe*.

## *Discussion*

We have isolated a novel member of the actin-associated protein family, PSTPIP, which binds to the PTP HSCF tyrosine phosphatase via an interaction between the proline-rich CTH domain of the PTP and the potential coiled-coil domain of the interacting protein. Like many other proteins associated with the cytoskeleton, PSTPIP is tyrosine phosphorylated in v-Src–transfected cells, and these phosphorylated residues appear to be substrates for the catalytic activity of the bound PTP HSCF. PSTPIP is localized to the cortical cytoskeleton, as well as in lamellipodia and it appears to migrate to the actin-rich cleavage furrow during cytokinesis. Overexpression of the protein in 3T3 cells induces long filopodial structures, consistent with a role for PSTPIP in the reorganization of the cytoskeleton. High level expression of the mammalian protein in the fission

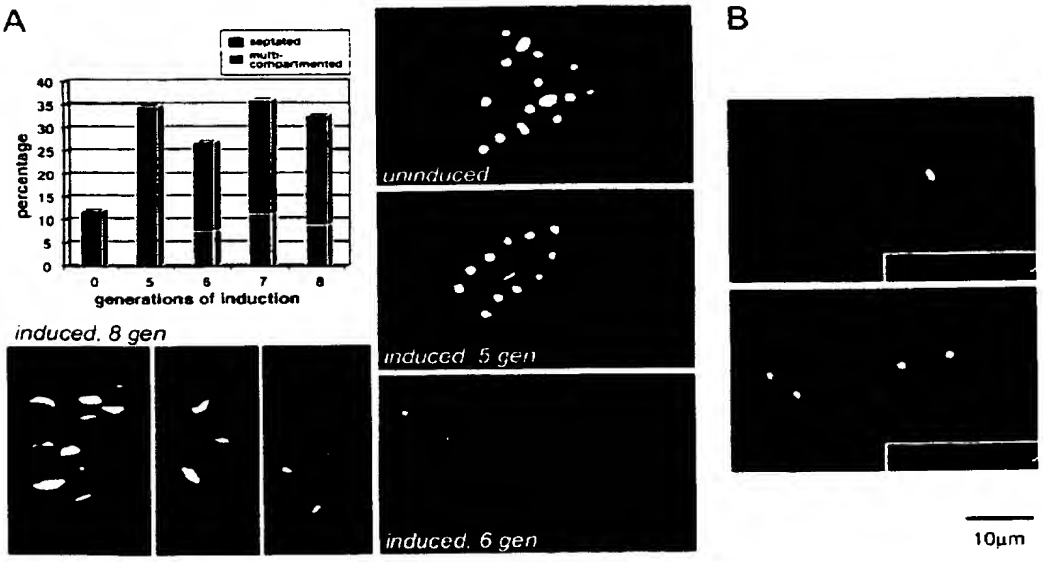


*Figure 10.* Overexpression of PSTPIP in *S. pombe*. (*A*) The graph illustrates the percentage of septated cells observed at the indicated generations following induction of PSTPIP expression by removal of thiamine. In this system, expression of genes under the control of the thiamine promoter begins at ~3.5 generations after removal of thiamine. The micrographs illustrate typical cells seen at each generation with their nuclei stained with DAPI and septa stained with calcofluor. Note the accumulation of septated cells at five generations and the appearance of multi-compartmented cells at six and eight generations. (*B*) Staining of PSTPIP overexpressing cells for PSTPIP with anti-FLAG antibody (*top*) and DAPI (*bottom*). Note the accumulation of the PSTPIP antigen in the cleavage furrow of the postmitotic cell. The inset shows accumulation of PSTPIP at the ends of the post-cleavage cells, a region previously shown to contain cortical actin. Bar, 10 μm.

yeast *S. pombe* resulted in [redacted]minant-negative inhibition of the completion of cytokinesis. These data suggest that PSTPIP is likely to be a cytoskeletal-associated protein, potentially involved with cytokinesis, whose physiological function is potentially regulated by its degree of tyrosine phosphorylation.

Analysis of the protein database for sequences with homology to PSTPIP suggests potential functions for this novel protein. Most of the sequences with significant homology to PSTPIP fall into the actin-associated family of proteins, and it is clear from the confocal studies reported here that PSTPIP is also associated with actin. While a number of other actin-interacting proteins, including myosin, fodrin, and spectrin, show homology to PSTPIP, the bulk of these homologies are within the SH3 domain, with little or no match in other regions of the protein. This is also true for cortactin (Wu et al., 1991), another protein that binds to the actin cytoskeleton in a similar manner, although there is additional weak homology in a small region of the coiled-coil domain in addition to the SH3 region. This is in contrast to the protein with the greatest degree of homology, the yeast *S.pombe* cdc15p, which shows significant sequence conservation in both the SH3 as well as the coiled-coil domains (Fankhauser et al., 1995). Cdc 15p is a highly phosphorylated protein that is absolutely required for the formation of the actin ring at the cleavage furrow of the postmitotic cell, and mutations in this protein result in an inability to assemble the actin ring over the postmitotic nucleus, thus resulting in multinucleate cells (Fankhauser et al., 1995). As with PSTPIP, cdc15p is localized to the cortical actin cytoskeleton until anaphase, when it migrates over the postmitotic nucleus and presumably mediates the reorganization of the cytoskeleton to the cleavage plane (Fankhauser et al., 1995; Simanis, 1995; Chang and Nurse, 1996). While the timing of PSTPIP migration to the cleavage furrow remains to be determined, its striking colocalization with the actin ring at this site during cytokinesis is analogous to what is observed with cdc15p (Fankhauser et al., 1995). In addition, the cdc15p is hyperphosphorylated until the onset of anaphase and the formation of the F-actin cytokinetic cleavage ring, when it becomes significantly dephosphorylated. Interestingly, the yeast protein regains its high state of phosphorylation at the conclusion of cell division, suggesting that phosphorylation regulates its association with the cleavage furrow. While the type of phosphorylation of cdc 15p has not yet been described, this suggests that tyrosine- and/or serine-threonine phosphatases must be involved with the regulation of the function of cdc 15p, and provides a mechanism whereby the binding and catalytic activity of a phosphatase such as PTP HSCF might function to control cytokinesis. Again, while the timing of tyrosine phosphorylation of PSTPIP during the cell cycle has yet to be determined, both the exact conservation of seven tyrosine residues between PSTPIP and cdc15p as well as the vanadate-sensitive tyrosine phosphorylation of the endogeneous interacting protein in Baf3 cells are suggestive of possible modulation of phosphotyrosine levels during the cell cycle. Thus, the sequence, cellular localization, and phosphorylation of both PSTPIP and cdc15p suggest that the mammalian protein is a potential homologue of cdc15p.

Phosphorylation, especially of serine and threonine residues, has [redacted] previously shown to play important roles in regulating events in cytokinesis and reorganization of the cytoskeletal (Mabuchi and Talcano-Ohmuro, 1990; Satterwhite et al., 1992; Tan et al., 1992; Egelhoff et al., 1993; Yamakita et al., 1994; Fishkind et al., 1995). To date, however, the possibility that tyrosine phosphorylation may play a role in these functions has been incompletely examined. The data reported in this paper suggest that the regulation of tyrosine phosphorylation on PSTPIP by PTP HSCF may play a role in aspects of cytoskeletal control including, possibly, cytokinesis. While the possible kinases involved in such tyrosine phosphorylation are numerous, the information described here, as well as elsewhere, suggests that a member of the Src family of tyrosine kinases may be involved with the phosphorylation of this interacting protein by either direct or indirect means. Two other PSTPIP-related proteins, cortactin and HSP 1, are both known to be tyrosine phosphorylated in v-Src–transformed cells, and cortactin, and potentially HSP 1 as well (Yamanashi et al., 1993), appear to interact with the cytoskeleton in a manner similar to PSTPIP (Wu et al., 1991; Dehio et al., 1995; Okamura et al., 1995; Vuori et al., 1995; Takemoto et al., 1996). In addition, a plethora of other proteins that are involved with the cytoskeleton are also tyrosine phosphorylated in v-Src–transformed cells (Schaller et al., 1993). Interestingly, the tyrosine phosphorylation of cortactin is also dramatically enhanced in cells isolated from mice deficient in the Csk kinase (Thomas et al., 1995), a tyrosine kinase which phosphorylates the COOH-terminal inhibitory tyrosine on c-Src, suggesting that cortactin is either a direct or indirect c-Src substrate in vivo. In addition, it has been demonstrated that HSP 1 can bind to the SH3 and SH2 domains of Src or Src family kinases in vitro, and it is also tyrosine phosphorylated by these kinases in vitro and in vivo (Takemoto et al., 1995, 1996). Although PSTPIP is only distantly related to cortactin and HSP 1, the tyrosine phosphorylation of this protein by v-Src in transfected cells may therefore have physiological relevance. In addition, previous data have demonstrated that c-Src associates with the focal adhesions and lamellipodia, as well as other actin-containing sites, consistent with the possibility that it could phosphorylate PSTPIP, which also localizes to these regions (Kaplan et al., 1994). Finally, v-Src is known to induce cytoskeletal changes in transformed cells, and it has been clearly shown that cortactin, an actin binding protein, becomes reoriented from the ends of the stress fibers to the podosomes of these v-Src–transformed cells, consistent with the possibility that phosphorylation of such actin-associated proteins might mediate changes in their cellular localization (Wu et al., 1991). Further analysis of the PSTPIP tyrosines phosphorylated in v-Src–transfected cells as well as by the endogenous tyrosine kinase will shed additional light on whether Src family kinases may mediate the phosphorylation of this interacting protein.

The use of dominant-negative forms of PTPs has previously been used to identify substrates for several enzymes, most notably PTP PEST (Garton et al., 1996) and the corkscrew PTP Src homology phosphatase-2 (SHP-2) (Herbst et al., 1996). In general, these studies have demonstrated that these dominant-negative mutants enhance the tyrosine phosphorylation of a surprisingly limited number

of substrates in vivo, in contrast to the relatively promiscuous behavior of these enzymes in vitro. In the case of the dephosphorylation of p130$^{cas}$ by PTP PEST, it appears that the substrate recognition is in large part due to the catalytic domain (Garton et al., 1996). The demonstration here that coexpression of two different dominant-negative forms of PTP HSCF mediates a dramatic increase in v-Src–induced PSTPIP tyrosine phosphorylation, together with the observation that this effect requires the COOH-terminal 24–amino acid homology domain, is thus consistent with several conclusions. The first is that these two proteins clearly interact in vivo, probably through the CTH domain, and the coiled-coil region interaction determined from the in vitro binding studies and the coprecipitation analyses (Figs. 5–7) supports such a physical interaction. This provides another example of the use of a noncatalytic region by a PTP to bring the catalytic domain in close proximity to the substrate (Tonks, 1993). In the case of the interaction described here, it appears that the juxtaposition of these proteins mediated by this interaction is required for substrate recognition, since the removal of the interaction domain from the PTP results in a complete lack of substrate recognition. The second is that it is possible that tyrosine-phosphorylated PSTPIP is an in vivo substrate for the PTP HSCF; and it also suggests that the enzyme inhibited by vanadate in the endogenous phosphotyrosine experiment in Baf3 cells, where both PSTPIP and PTP HSCF are expressed, is likely to be PTP HSCF (Cheng et al., 1996). While it is possible to test such a hypothesis by coprecipitation studies, we have found that the level of PTP HSCF expressed by Baf3 cells is insufficient for detection by Western blotting. Finally, if we assume that the mutant forms of PTP HSCF are endowed with the same degree of substrate specificity that has been found with other dominant-negative PTPs (Dixon, 1995; Garton et al., 1996; Herbst et al., 1996), then the v-Src cotransfection studies further suggest that either Src or a related family member may be kinases that are involved with the tyrosine phosphorylation of PSTPIP in vivo in nontransfected cells.

The v-Src–mediated tyrosine phosphorylation of PTP HSCF, together with the demonstration that dominant-negative forms of the enzyme induce a hyperphosphorylated state, strongly suggest that this PTP mediates its own autodephosphorylation. As expected, while the dominant-negative effect of the PTP on PSTPIP phosphorylation requires the COOH-terminal 24–amino acid homology domain, the dominant-negative hyperphosphorylation of the PTP does not require this region, further supporting the hypothesis that the interaction between PSTPIP and PTP HSCF is required for substrate recognition. Other PTPs, including SHP-1 (Bouchard et al., 1994; Lorenz et al., 1994), SHP-2 (Lechleider et al., 1993; Vogel et al., 1993; Feng et al., 1993, 1994; Stein-Gerlach et al., 1995), and PTPα (den Hertog et al., 1994) also appear to be tyrosine phosphorylated in response to a number of different stimuli. Interestingly, like PTP HSCF, SHP-2 appears to modulate its own phosphotyrosine levels (Stein-Gerlach et al., 1995), and these levels appear to modestly modulate enzymatic activity (Vogel et al., 1993). SHP-1 (Lorenz et al., 1994) and SHP-2 (Feng et al., 1994) are tyrosine phosphorylated in vivo by two Src family kinases, Lck and v-Src, respectively; this is consistent with results reported here that demonstrate tyrosine phosphorylation of PTP HSCF by v-Src. Moreover, tyrosine phosphorylation of SHP-2 and PTPα both result in the association of the GRB2 adaptor protein (den Hertog et al., 1994; Vogel et al., 1996). Together, these data suggest that modification of tyrosines on PTPs is a potential regulatory mechanism, and it should prove interesting to examine the role of phosphatase tyrosine phosphorylation in the system described here.

The nature of the high affinity binding between the proline-rich CTH domain and the coiled-coil region is reminiscent of that previously described for the SH3–proline-rich core interaction (Pawson, 1995; Feng et al., 1995). In this latter case, proline helices induce the formation of highly structured small peptide domains that bind with relatively high affinity and specificity to the binding pocket of the SH3 domain, and various interactions, including salt bridges, mediate the specificity and directionality of peptide binding (Feng et al., 1995). Analysis of the proline-rich CTH domains of three PEST PTPs, all of which appear to inhibit the PSTPIP-PTP HSCF binding interaction with similar 50% inhibitory concentrations (<1 μM), reveals that they share a proline-rich core region that would be predicted to form a proline helix similar to that seen for SH3 binding sites (Matthews et al., 1992; Yang et al., 1993; Cheng et al., 1996). This region contains a number of charged residues, and it appears that the potential helical nature of this domain positions these residues in an appropriate binding conformation for interaction with a site within the coiled-coil domain (Dowbenko, D., and L. Lasky, unpublished observations). Because all of the PEST PTPs are predicted to bind to PSTPIP via this proline-rich region, it is possible that the interacting protein's phosphotyrosine content is modulated by different PEST PTPs in different cell types. Along these lines, it is interesting to note that the only hyperphosphorylated protein observed in COS cells transfected with dominant-negative (Asp–Ala) PTP PEST was p130$^{cas}$ (Garton et al., 1996). These results suggest that, if PSTPIP is highly expressed in COS cells, it is either not tyrosine phosphorylated or is not a substrate for this PTP in this cell line.

The mechanism by which PSTPIP migrates from the cortical actin, lamellipodia and stress fiber regions in resting cells to the cytokinetic cleavage furrow in dividing cells can only be speculated upon (Strome et al., 1993; Fishkind et al., 1995). One possibility is that this protein binds tightly to actin, and when the actin is reoriented to the cleavage plane, the PSTPIP accompanies it passively (Cao et al., 1990*a*,*b*; Fishkind et al., 1993). However, experiments in yeast where cdc15p is deleted revealed that cortical actin did not migrate to the cleavage plane in the absence of this protein, suggesting that cdc15p actively traverses to this site and mediates the assembly of the actin ring (Fankhauser et al., 1995; Simanis, 1995). Thus these data suggest that if PSTPIP is a mammalian homologue of cdc15p, that dominant-negative mutants in this protein should affect the assembly of actin at the vertebrate cleavage furrow, and we are currently testing this possibility. Interestingly, it appears that deletion mutants of cdc15p that lack the SH3 domain are incapable of rescuing the cdc15 mutants, suggesting a critical role for this COOH-terminal domain in assembling the cytokinetic actin ring (Fankhauser et al.,

1995). It will therefore be important to determine the proteins which bind to this domain in PSTPIP, since they may provide additional insights into its physiological function.

A possible mechanism by which PSTPIP functions is suggested by the results of overexpression studies in murine 3T3 cells. The extended filopodial structures in many of these transfected cells are consistent with the possibility that the unregulated expression of the protein mediates an ectopic and organized assembly of actin filaments, thus resulting in a cellular protrusion containing PSTPIP and F-actin. While the striking level of lysine residues in the predicted coiled-coil domain of this protein is consistent with previously described actin binding sites (Vandekerckhove, 1990; Friederich et al., 1992), we have been unable to demonstrate a stable association between PSTPIP and F-actin in vitro (Wu, Y., and L.A. Lasky, unpublished data). Interestingly, many of the transfected cells contained a single filopodial-like structure, suggesting that this morphological feature is rapidly formed and is likely to have a negative influence on cell viability. The apparent small size of many of these cells suggests that this actin-containing spike is formed in the absence of plasma membrane synthesis, also consistent with a rapid formation of the structure. The evident heterogeneity in penetrance of this morphological entity may either be due to diverse expression levels or differences in posttranslational modifications of the transfected proteins. If these suppositions are correct, then it would appear that PSTPIP may play a role in the rapid assembly of a highly organized F-actin–containing structure.

The results of overexpression of mammalian PSTPIP in exponentially growing *S. pombe* provide support for a role for this protein in cytokinesis. Fission yeast cells grow mainly by elongation at their tips, and divide by binary fission after forming a centrally placed actin-rich septum. In *S. pombe*, F-actin is seen as patches or dots at sites of cell growth or division. During interphase, it is found at the growing ends of the cell, and, after the onset of mitosis, it relocates to form an equatorial ring whose position anticipates the site of septum formation (Marks and Hyams, 1985; for review see Robinow and Hyams, 1989). At the end of mitosis, when the daughter nuclei are well separated and the spindle begins to break down, the septum grows inwards from the cell cortex. Secondary septa are formed on either side of the primary septum, which is subsequently dissolved to effect cell separation. F-actin is then relocated to the old (preexisting) end of the cell, from which growth resumes. Some of the proteins that control the onset of septum formation and cytokinesis in fission yeast have been identified. The products of the *cdc3*, *cdc4*, *cdc8*, *cdc12*, *cdc15*, and *rng2* genes are required for actin rearrangement and/or to stabilize the actin ring (Nurse et al., 1976; Balasubramanian et al., 1992, 1994; Fankhauser et al., 1995; McCollum et al., 1995; Chang et al., 1996). Immunofluorescence studies have shown that the products of the *dmf1*, *cdc3*, *cdc4*, *cdc8*, and *cdc15* genes are associated with the medial ring (Balasubramanian et al., 1992, 1994; Fankhauser et al., 1995; McCollum et al., 1995), a cellular localization similar to that observed for PSTPIP in both mammalian cells and yeast. At the end of mitosis, the *cdc7* kinase and the activities of Cdc11p and Cdc14p are required for septation (Nurse et al., 1976; Fankhauser and Simanis, 1994). The plo1 kinase appears to be required for actin ring formation and septation, and can induce septum formation from G1 and G2, if expressed at a very high level. These data are consistent with the hypothesis that protein phosphorylation is involved with the regulation of cytokinesis. The dominant-negative phenotype observed here is similar to that seen in fission yeast that are null for the expression of the calcineurin-like phosphatase, ppb 1, although this is presumably a serine/threonine phosphatase, while PSTPIP binds to a tyrosine phosphatase (Yoshida et al., 1994). Nevertheless, the data are consistent with the possibility that PSTPIP titrates out an important component of the *S. pombe* cytokinetic machinery, with the result that cleavage is inhibited.

The results reported here describe PSTPIP, a novel member of the cytoskeleton-associated protein family, which is a substrate for the PTP HSCF. The homology of PSTPIP with the fission yeast cdc15 protein, together with the demonstration of in vivo tyrosine phosphorylation of the interacting protein, association with the cortical cytoskeleton and cellular cleavage furrow, the induction of cytoskeletal alterations, and the dominant-negative inhibition of *S. pombe* cytokinesis are all consistent with the possibility that PSTPIP is a mammalian homologue of the yeast cleavage furrow regulatory protein. Because this protein interacts with and is an enzymatic substrate for the PTP HSCF, this hypothesis suggests that the control of tyrosine phosphorylation of PSTPIP by this PTP may provide for a novel mechanism for the control of cleavage furrow formation during cytokinesis.

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